JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Detection of Transgenic and Endogenous Plant DNA in Digesta and Tissues of Sheep and Pigs Fed Roundup Ready Canola Meal

RANJANA SHARMA,[†] DANA DAMGAARD,[†,||] TREVOR W. ALEXANDER,[†] MIKE E. R. DUGAN,[‡] JENNIFER L. AALHUS,[‡] KIM STANFORD,[§] AND TIM A. MCALLISTER*,[†]

Agriculture and Agri-Food Canada Research Centres, Lethbridge, Alberta, Canada T1J 4B1, and Lacombe, Alberta, Canada T4L 1W1, and Alberta Agriculture, Food and Rural Development, Lethbridge, Alberta, Canada T1J 4V6

The persistence of plant-derived recombinant DNA in sheep and pigs fed genetically modified (Roundup Ready) canola was assessed by PCR and Southern hybridization analysis of DNA extracted from digesta, gastrointestinal (GI) tract tissues, and visceral organs. Sheep ($n = 11$) and pigs ($n = 36$) were fed to slaughter on diets containing 6.5 or 15% Roundup Ready canola. Native plant DNA (high- and low-copy-number gene fragments) and the *cp4 epsps* transgene that encodes 5-enolpyruvyl shikimate-3-phosphate synthase were tracked in ruminal, abomasal, and large intestinal digesta and in tissue from the esophagus, rumen, abomasum, small and large intestine, liver, and kidney of sheep and in cecal content and tissue from the duodenum, cecum, liver, spleen, and kidney of pigs. High-copy chloroplast-specific DNA (a 520-bp fragment) was detected in all digesta samples, the majority (89−100%) of intestinal tissues, and at least one of each visceral organ sample (frequencies of 3−27%) from sheep and swine. Low-copy *rubisco* fragments (186- and 540-bp sequences from the small subunit) were present at slightly lower, variable frequencies in digesta (18−82%) and intestinal tissues (9−27% of ovine and 17−25% of porcine samples) and infrequently in visceral organs (1 of 88 ovine samples; 3 of 216 porcine samples). Each of the five *cp4 epsps* transgene fragments (179−527 bp) surveyed was present in at least 27% of ovine large intestinal content samples (maximum = 64%) and at least 33% of porcine cecal content samples (maximum = 75%). In sheep, transgene fragments were more common in intestinal digesta than in ruminal or abomasal content. Transgene fragments were detected in 0 (esophagus) to 3 (large intestine) GI tract tissues from the 11 sheep and in 0−10 of the duodenal and cecal tissues collected from 36 pigs. The feed-ingested recombinant DNA was not detected in visceral tissues (liver, kidney) of lambs or in the spleen from pigs. Of note, however, one liver and one kidney sample from the pigs (different animals) were positive for a 278-bp fragment of the transgenic *cp4 epsps* (denoted F3). Examination of genomic libraries from these tissues yielded no conclusive information regarding integration of the fragment into porcine DNA. This study confirms that feed-ingested DNA fragments (endogenous and transgenic) do survive to the terminal GI tract and that uptake into gut epithelial tissues does occur. A very low frequency of transmittance to visceral tissue was confirmed in pigs, but not in sheep. It is recognized that the low copy number of transgenes in GM feeds is a challenge to their detection in tissues, but there was no evidence to suggest that recombinant DNA would be processed in the gut in any manner different from endogenous feed-ingested genetic material.

KEYWORDS: ***cp4 epsps*; Roundup Ready; genetically modified canola; recombinant DNA; lambs; swine; biotechnology; PCR**

INTRODUCTION

The adoption of genetically modified (GM) plants for animal and human consumption has increased over the past few years. Although regulatory agencies have approved the use of GM crops (*1*), consumers are still concerned regarding the possible transfer of transgenic DNA from feed to livestock and livestock-derived products that are consumed by humans. Studies that examine the fate of recombinant feed DNA in the gastrointes-

* Author to whom correspondence should be addressed [telephone (403) 317-2240; fax (403) 382-3156; e-mail mcallister@agr.gc.ca].
[†] Lethbridge Research Centre.
[||] Present address: Norwest Labs, Lethbridge, Alberta, Canada T1J 4H1.
[‡] Lacombe Research Centre.
[§] Alberta Agriculture, Food and Rural Development.

10.1021/jf052459o CCC: $33.50 Published 2006 by the American Chemical Society
Published on Web 02/10/2006

tinal (GI) tract of livestock and its potential absorption and transfer to other tissues are essential to fully address these safety concerns. At present, a number of GM plants are approved for cultivation in Canada and the United States, including canola, corn, cotton seed, flax, lentils, potato, rice, soybean, squash, sugar beet, tomato, and wheat. In the European Union, 14 varieties have been approved, of which 8 are permitted to be used for food and feed purposes (*2*).

To date, most studies examining the fate of transgenic DNA in feed crops have focused on insect-resistant Bt-corn or Bt-soybean. However, the majority of canola (i.e., low glucosinolate, low erucic acid rapeseed) grown in Canada is genetically modified for tolerance of the herbicide glyphosate. This is achieved by expression of the *cp4 epsps* transgene that encodes 5-enolpyruvyl shikimate-3-phosphate synthase. This investigation focused on meal prepared from GM (Roundup Ready) canola, which is used extensively as a protein source in the diets of both ruminant and monogastric livestock.

Absorption of transgenic DNA from GM feed would require that the transgenic DNA remain intact and stable as it passes through the GI tract. Previous research in our laboratory (*3*) has shown that in the presence of ruminal fluid, recombinant DNA is rapidly degraded (as early as 10 min). Despite this, transgenic DNA has been detected from ruminal and duodenal digesta, suggesting that transgenes are not completely degraded in the rumen (*4*). In mice, fragments of ingested DNA have been shown to persist in the GI tract, to cross the intestinal wall and subsequently be detected in leukocytes, spleen, and liver cells (*5*, *6*), and to move across the placenta to the fetus (*7*). A recent study using gnotobiotic rats indicates that ingested DNA may persist in the lower GI tract and be available for uptake by intestinal bacteria (*8*). In contrast, other studies have not detected recombinant DNA or protein in the tissues or products of poultry (*9*−*12*) or livestock (*10*, *13*−*18*) fed transgenic feed. However, small fragments (fewer than 300 bp) of chloroplast DNA have been detected from poultry (*10*) and swine tissues (*17*, *19*). Recent human intestinal simulations and studies with ileostomists have further indicated that transgene from GM soy and maize may survive passage through the small intestine (*20*); however, Netherwood et al. (*21*) reported that all transgenic DNA was degraded within the colon.

To evaluate the safety of GM feeds, the stability and persistence of transgenes in the gastrointestinal tract of animals must be determined. The present study was conducted to investigate and compare the fate of endogenous and transgenic plant DNA within the digestive tract, GI tract tissues, and organ tissues from ruminant and monogastric livestock (lambs and swine) fed RR canola meal throughout the growing period. The study was also intended to provide additional data on optimizing sensitive and reproducible assays for the detection of fragments of transgenic DNA in animals fed diets containing RR canola.

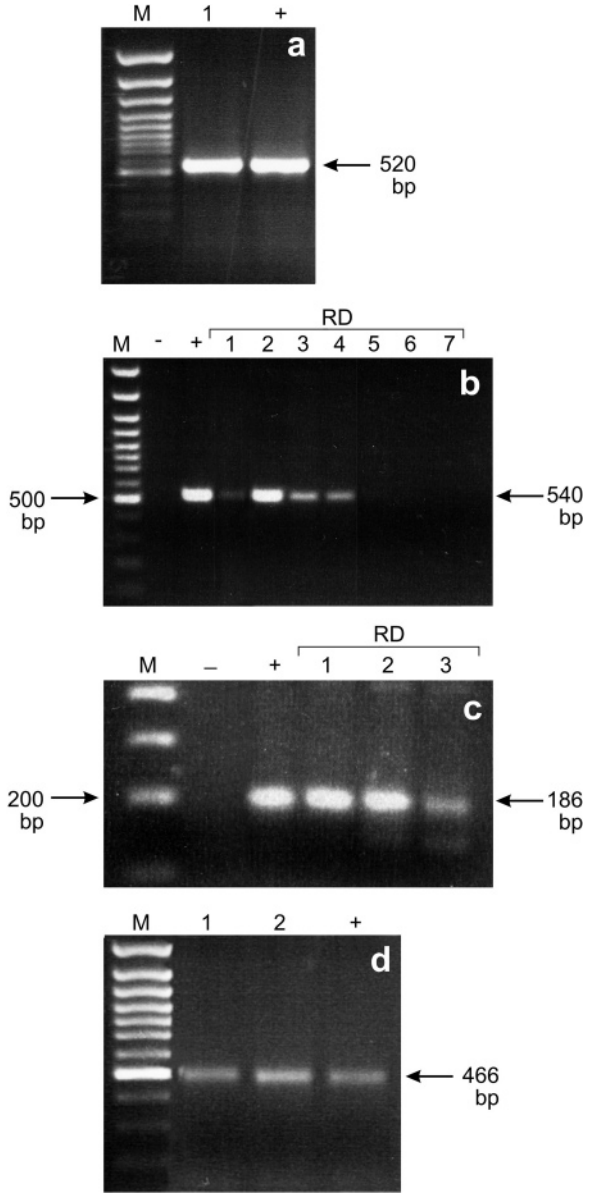

Figure 1. Confirmation of amplification of endogenous plant (**a**–**c**) and bacterial (**d**) DNA in digesta samples from lambs (representative data): (**a**) amplification of high-copy chloroplast-specific DNA fragment Cp; (**b**) amplification of *rubisco* fragment *rbc* LF; (**c**) amplification of *rbc* SF; (**d**) amplification of bacterial 16S rDNA from ruminal (lane 1) and abomasal (lane 2) digesta. In each panel, lane M contains a 100-bp DNA ladder, – is a negative control (no DNA template), and + is a positive control [i.e., DNA extracted from Roundup Ready canola leaf in (**a**–**c**) or DNA from *E. coli* in (**d**)]. In (**a**–**c**), numbered lanes contain DNA extracted from ruminal digesta (RD) of individual lambs.

MATERIALS AND METHODS

All animals in this study were cared for according to the standards set by the Canadian Council on Animal Care (*22*).

Diet Preparation and Feeding Trials. Transgenic canola (Roundup Ready, RR) and a near-isogenic parental line from which RR was developed were provided by Monsanto Co. (St. Louis, MO), and meals were prepared from them at the Texas Agriculture Experiment Station, Texas A&M University (College Station, TX). The presence of the 1.3-kb *cp4 epsps* gene in the RR meal, and its absence in the parental meal, were confirmed in our laboratory during a related study with these canola lines (*23*, Figure 2).

Diets for sheep (*24*) were prepared at the Lethbridge Research Centre for a growth performance study conducted with early-weaned, individually fed Canadian Arcott lambs. The diets contained canola meal (6.5%, DM basis) either from parental (control) or RR canola, or from two other commercially available lines, and were pelleted at 100 °C following mixing of all ingredients. To preclude contamination during diet preparation, the control (PAR) diet was mixed before the RR diet, both as single batches, and the pelleting system was flushed with 100 kg of steam-rolled barley before, between, and after batches. Each diet was assessed by Polymerase Chain Reaction (PCR) to confirm the absence (in PAR) or presence (in RR) of the *cp4 epsps* transgene. The lambs were fed the experimental diets ($n = 15$) for 6−8 weeks, from initial body weights (BW) of 21.5 ± 1.0 kg to final BW of ≥45 kg (weighed weekly) and then were slaughtered in a research abattoir (Lacombe, AB).

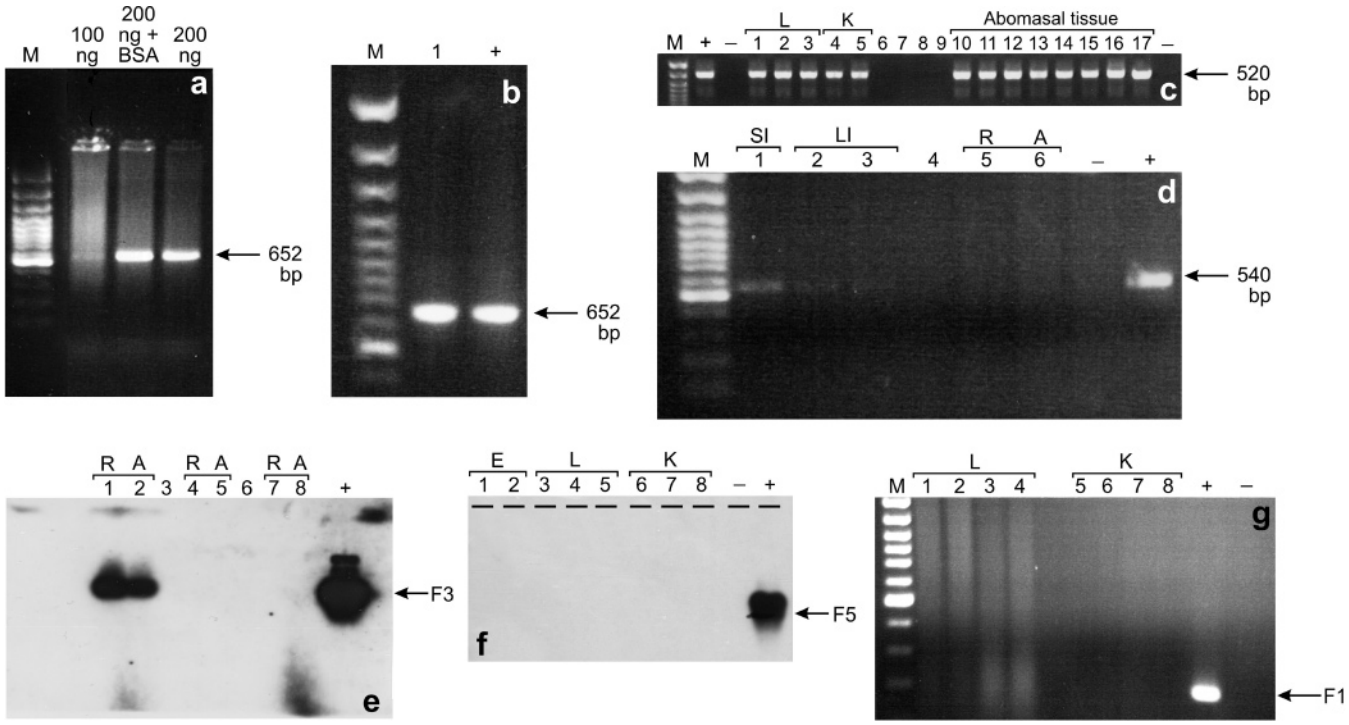

Figure 2. Representative electrophoretic gels and Southern hybridizations of PCR analyses of digesta and tissues from 11 sheep fed diets containing Roundup Ready canola: (**a**) detection of ovine control DNA in abomasal tissue during preliminary analyses to standardize PCR conditions (100 vs 200 ng of template DNA; including vs excluding 5 μg of BSA); (**b**) amplification of ovine growth factor DNA, demonstrating PCR readiness of DNA extracted from ovine liver tissue (lane 1) [DNA isolated from sheep blood was used as positive control (lane +)]; (**c**) detection of a 520-bp chloroplast-specific DNA fragment (Cp) in liver (L; lanes 1–3), kidney (K; lanes 4 and 5), and abomasal tissues (lanes 10–17) from the 3, 2, and 8 sheep, respectively, that were positive for Cp in the tissues specified (see **Table 2**) (lanes 6–9 are empty); (**d**) detection of a 540-bp *rbc* LF fragment in small intestinal (SI; lane 1) and large intestinal (LI; lanes 2 and 3) tissues from the sheep (SI, 1 of 11; LI, 2 of 11) that tested positive (see **Table 2**) and representative negative findings for *rbc* LF in ruminal (R) and abomasal (A) tissues [lane 4 is empty; very faint bands evident in lanes 2 and 3 were confirmed by Southern blotting and hybridization (data not shown)]; (**e**) representative positive (lanes 1 and 2) and negative (lanes 4, 5 and 7, 8) findings for detection of *cp4 epsps* transgene fragment F3 in ruminal (R) and abomasal (A) tissues from three sheep by Southern blotting and hybridization (lanes 3 and 6 are empty); (**f**) representative negative findings for detection of *cp4 epsps* fragment F5 in ovine esophageal (E; lanes 1 and 2), liver (L; lanes 3–5), and kidney (K; lanes 6–8) tissues by Southern blotting and hybridization; (**g**) representative negative findings for detection of *cp4 epsps* fragment F1 in liver (L) and kidney (K) tissues from four sheep. In each panel, lane M contains a 100-bp DNA ladder. The negative control lanes (–) contain no template DNA.

Grower and finisher diets for pigs were prepared at Lacombe, AB. As with diets for lambs, the PAR diet was mixed prior to the RR diet, and system flushing was conducted with ~350 kg of wheat middlings before, between, and after experimental diets. The diets were formulated according to NRC guidelines (*25*) to be isocaloric and have equal ileal lysine apparent digestibilities and to contain 7.5% canola meal (as-fed) in grower diets and 15% canola meal in finisher diets (**Table 1**).

Piglets for a performance trial conducted with PAR and RR diets (unpublished data) were obtained by crossing Genex Manor Hybrid (Large White × Landrace) sows with Yorkshire boars from the Lacombe Research Centre herd, batch farrowing every 2 weeks. All healthy pigs from each batch farrowing were moved to one of three research rooms, with original penning groups maintained. The piglets were allowed at least 1 week for adaptation to the new environment, following which the PAR diet was introduced and fed until pen weight averaged 30 ± 3 kg. At that time, pens were assigned randomly within gender to be fed PAR or RR, with a total of 36 pigs per treatment. The pigs were given ad libitum access to feed and water for the duration of the study. They were fed a grower diet (PAR or RR) to an average pen weight of 60 ± 3 kg and then a finisher diet (continuing on PAR or RR) until slaughter at average weight of 108 ± 5 kg.

Tissue Collection and Transport. Tissue and digesta samples were collected at slaughter (those fed PAR preceding those fed RR) from 11 of the 15 lambs (randomly selected) in each group. Samples (10−15 g) were collected from the esophagus (immediately proximal to esophageal orifice), rumen (ventral sac), abomasum (adjacent to pylorus), small intestine (duodenum), and large intestine (immediately proximal to small intestine). Approximately 15 g of digesta were collected from the rumen, abomasum, and large intestine from the same site as tissue samples. Preliminary analyses of individual and pooled samples from PAR-fed lambs were consistently negative for transgene; thus, investigation was continued and reported herein on tissues from RR-fed lambs only.

From each of the 36 pigs fed RR canola meal, 20-g samples were collected at slaughter from the kidney (proximal portion of the cortex), spleen (anterior end), liver (distal end of central lobe), duodenum (immediately proximal to the stomach), and cecum (immediately proximal to small intestine). Approximately 15 g of digesta from cecum was also collected for DNA analysis. Digesta and the kidney, spleen, and liver tissues were collected in separate areas of the research abattoir to avoid cross-contamination from digesta to organ tissue.

Kidney tissue from a pig fed diet PAR was collected and processed identically, including PCR extraction, to the samples from RR-fed pigs. As well, ovine DNA was extracted from blood collected (by jugular venipuncture; stored at −20 °C in a Vacutainer tube) from a sheep that was housed at the Lethbridge Research Centre, but unrelated to the present study. These extractions were conducted to confirm that the procedures used in this study did yield PCR-quality (i.e., amplifiable) DNA template and to provide DNA for known positive controls (with appropriate primer pairs) for inclusion in each PCR setup to confirm suitability of PCR conditions for amplification.

All tissue samples were rinsed thoroughly with 1× phosphate-buffered saline. Digesta and tissue samples were collected into sealable plastic bags prelabeled with animal identification, date, and sample type,

Table 1. Ingredients and Composition of Experimental Diets Containing Parental (PAR) or Roundup Ready (RR) Canola Meal, Fed to Sheep or Pigs

	diets for sheep		diets for pigs			
			grower diets		finisher diets	
item	PAR	RR	PAR	RR	PAR	RR
ingredients (kg tonne^{-1}, as-fed)						
barley grain	756	756	250.0	250.0	696.7	695.9
wheat grain			485.9	487.9	100.0	100.0
alfalfa, sun-cured	126	126				
canola meal (PAR)	65	0	75.0	0	150.0	0.0
canola meal (RR)	0	65	0	75.0	0.0	150.0
soybean meal			127.5	125.0		
canola oil	5.0	5.0	30.5	31.0	29.0	29.5
beet molasses	20	20				
calcium carbonate	16	16	7.50	7.50	7.50	7.50
sheep mineral[a]	7	7				
Maxi-Pel[b]	5	5				
vitamin ADE[c]	0.250	0.250				
Deccox[d]	0.132	0.132				
dicalcium phosphate (21%)			12.0	12.0	7.50	7.50
sodium chloride			5.07	5.07	5.08	5.08
lysine HCl (98%)			2.50	2.50	1.57	1.44
Tylan 40			1.25	1.25	0.25	0.25
threonine-L			0.57	0.57	0.11	0.07
choline chloride (60%)			0.50	0.50	0.50	0.50
swine trace mineral mix[e]			1.00	1.00	1.00	1.00
swine vitamin mix[f]			0.75	0.75	0.75	0.75
composition (%, as-fed)						
dry matter	89.09	90.55	88.3	88.2	88.4	88.6
organic matter	82.45	83.75	nd[g]	nd	nd	nd
crude protein	14.92	15.67	20.1	18.5	15.6	15.9
crude fat	2.36	2.45	4.8	5.4	5.2	5.0
neutral detergent fiber	24.40	24.53	nd	nd	nd	nd
acid detergent fiber	11.92	11.63	nd	nd	nd	nd
calcium	nd	nd	0.64	0.79	0.59	0.66
phosphorus	nd	nd	0.69	0.71	0.60	0.65
lysine[h]	nd	nd	0.83	0.83	0.60	0.60
threonine[h]	nd	nd	0.51	0.51	0.37	0.37
DE[i] (MJ kg^{-1}; calculated)	nd	nd	14.64	14.64	13.81	13.81

[a] Containing (g kg^{-1}): NaCl, 931; Fe, 12.5; K, 12.5; Mg, 12.5; Mn, 9.4; Zn, 9; Cu, 1.3; Se, 0.03. [b] Feed pellet binder (Mountain Minerals Ltd., Lethbridge, AB, Canada). [c] Containing (IU g^{-1}): vitamin A, 10000; vitamin D, 1250; vitamin E, 10. [d] Decoquinate, 60 g kg^{-1} (Rhône-Poulenc Canada, Mississauga, ON, Canada). [e] Containing (g kg^{-1}): Zn, 80; Fe, 64; Mn, 40; Cu, 9.6; Co, 1; I, 0.42; Se, 0.12. [f] Containing (kg^{-1}): vitamin A, 5 400 000 IU; vitamin D_3 600 000 IU; vitamin E, 30000 IU; niacin, 15000 mg; pantothenic acid, 9000 mg; riboflavin, 3.350 g; thiamin, 1000 mg; folic acid, 650 mg; biotin, 100 mg; manadione, 100 mg; vitamin B_{12}, 10 mg. [g] nd, not determined. [h] Calculated, apparent ileal digestible. Other components, as-fed, analyzed concentrations. [i] DE, digestible energy.

and the entire bag was immediately frozen in liquid nitrogen, sealed, and transferred directly to storage at −80 °C or shipped in liquid nitrogen from Lacombe to the Lethbridge Research Centre for storage until processing for DNA extraction. Time elapsing between kill time and immersion of plastic bags for freezing did not exceed 30 min. At Lethbridge, frozen samples in the original sealed plastic bags were impact-fractured into subsamples (2−3 g each) with surfaces freshly exposed from the interior of the collected tissue. Three subsamples from each collected tissue were selected and returned separately to storage at −80 °C.

Extraction of DNA. Care was taken during DNA extraction (and PCR) to minimize the potential for cross-contamination of samples. Aseptic techniques including autoclaving and/or treatment of equipment with 10% (v/v) bleach solution (0.4−0.6% sodium hypochlorite) were used throughout the protocols, and three separate work locations were utilized for sample grinding/processing, conduct of PCR, and post-PCR processing. Extracted DNA was stored in a location distinct from the extraction site. Centrifuges were cleaned routinely with DNA-Off decontamination solution (Mandel Scientific, Guelph, ON), separate pipets were dedicated for DNA extraction and PCR, and sterile aerosol-resistant tips and disposable RNase/DNase-free plasticware were used.

Canola Leaf Tissue. Parental and Roundup Ready (RR) canola seed provided by Monsanto Co. was germinated and grown in the phytotron facility at the Lethbridge Research Centre to provide leaf tissue. The DNeasy plant Mini Kit (Qiagen Inc., Mississauga, ON) was used to extract DNA from parental and RR leaves for negative or positive controls for PCR (as indicated).

Seeds, Meals, and Diets. DNA from parental and RR canola seed, meals, and diets was extracted using a CTAB extraction procedure modified as described by Alexander et al. (*23*).

Ovine and Porcine Tissues. DNA from lamb blood was extracted using the DNA blood mini kit (Qiagen). Two of the three replicate ovine and porcine tissue subsamples were subjected to separate DNA extractions using the Wizard genomic DNA extraction kit (Promega Ltd., Madison, WI). The manufacturer's protocol was modified slightly as follows: the frozen subsamples (2 g) were ground in liquid nitrogen before addition of nuclei lysis buffer (supplied in the kit), and the lysates were incubated overnight at 65 °C. Following addition of 25 μL of RNase solution (100 mg/mL; Roche, Laval, QC), the lysates were incubated for a further 40 min at 37 °C, following which 5 mL of the supplied protein precipitation solution was added and the content of each tube was mixed gently by inversion. Samples were incubated for 30 min at 4 °C and then centrifuged at 13000*g* for 10 min. Supernatant fluids were transferred to clean tubes, and DNA was precipitated with 0.8 volume of 2-propanol. The DNA pellet thus obtained was washed twice with 70% (v/v) ethanol and dissolved in 500 μL of DNA rehydration solution.

Digesta. The DNA from duplicate digesta subsamples (2 g) collected from lambs was extracted as described by Sharma et al. (*26*). The QIAmp Stool kit (Qiagen) was used to extract DNA from duplicate cecal content subsamples from pigs (2 g), with the manufacturer's protocol modified as follows: After homogenization of sample in liquid nitrogen, samples were resuspended in the ASL buffer from the kit and incubated at 65 °C for 4 h with gentle shaking. The lysate was

Table 2. Primers Used in PCR for Detection of Plant, Animal, and Bacterial DNA

specificity	primer name	amplicon size (bp)	target gene/region	ref
plant (endogenous)				
rbc LF[a]	LF/LR	540	*rubisco*	*23*
rbc SF[a]	SF/SR	186	*rubisco*	*28*
Cp	CpF/CpR	520	chloroplast DNA	based on *29*
plant (transgenic)[b]				
F1	PF2/ER3	179	promoter/chloroplast transit peptide	*28*
F2	PF2/ER1	527	promoter/*cp4 epsps*[c]	*28*
F3	EF5/ER5	278	within *cp4 epsps*	*28*
F4	EF2/ER2	270	within *cp4 epsps*	*28*
F5	EF2/TR	420	*cp4 epsps*/terminator	*28*
F6	EF1/ER1	1363	*cp4 epsps* (entire gene)	*23*
animal				
ovine	OF/OR	652	growth factor	*31*
porcine	PF/PR	532	growth factor	*30*
bacterial				
Bc	BF/BR	466	16S rDNA	*32*

[a] *rbc* LF, large fragment from gene encoding ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) small subunit; *rbc* SF, small fragment from gene encoding rubisco small subunit; Cp, chloroplast-specific DNA. [b] Fragments F1, F2, and F5 are construct-specific; F3 and F4 are gene-specific; F6 represents the entire 1.3-kb *cp4 epsps*. [c] *cp4 epsps* is the transgene encoding 5-enolpyruvylshikimate-3-phosphate synthase that confers glyphosate tolerance (the Roundup Ready characteristic) upon canola.

centrifuged at 12000*g* for 5 min. Twice the recommended volume of Proteinase K was used. Samples were incubated for a further 30 min after the addition of AL buffer. Two washes with AW2 buffer were carried out followed by elution of the DNA.

Extraction Protocols. All extractions were monitored routinely with negative controls (buffer without tissue) as surveillance for any cross-contamination having arisen during routine sample grinding and processing. Extraction efficiencies were ascertained regularly by adding known amounts of DNA (100 pg of RR leaf DNA) to the test samples prior to DNA extraction. In the infrequent instance when DNA extraction did not appear to be successful, it was repeated with the third replicate subsample fractured from the tissue.

The purity and quantity of DNA were determined prior to PCR using an Ultraspec 3000 spectrophotometer (Pharmacia Biotech Ltd., Cambridge, U.K.) and a VersaFluor fluorometry system (Bio-Rad, Hercules, CA). Extracted genomic DNA (5 μL) was assessed routinely by electrophoresis on 0.7% (w/v) agarose prior to PCR.

Polymerase Chain Reactions. PCR was conducted in duplicate on each extracted DNA sample. When amplification of target fragments was ambiguous (positive in one replicate; negative in the second), the PCR was repeated to confirm the status of the DNA with regard to the presence or absence of the target fragment. Each PCR run included (i) a negative control containing no template DNA and (ii) appropriate positive controls (100 ng), comprising parental and/or transgenic canola leaf DNA as controls for plant DNA, *Escherichia coli* DNA as a control for bacterial DNA, and/or ovine (blood) or porcine (kidney) DNA from animals fed PAR diet as a control for animal DNA. As well, PCR reactions were monitored for false negatives by spiking the sample DNA extracts with known amounts of DNA from RR canola.

Primers for bacterial, ovine, and bovine DNA controls, and those for detecting the *cp4 epsps* transgene, were synthesized at the Lethbridge Research Centre on a DNA/RNA synthesizer (Applied Biosystems, Foster City, CA) and/or purchased from Invitrogen Life Technologies (Burlington, ON). All PCR amplifications were performed on a Peltier thermal cycler (PTC100; MJ Research Inc., Watertown, MA). Each reaction mix (50 μL) contained the following (final concentrations): dNTP mix (0.2 mM), forward and reverse primer (each at 0.5 μM), $MgCl_2$ (1.5 mM), and 2.5 units of Platinum *Taq* Polymerase (Invitrogen) in 1× PCR buffer, as well as 5 μg of bovine serum albumin (BSA) to increase assay robustness (*27*). For DNA isolated from plant sources (leaves and diets), 100 ng of DNA template was used. For organ tissues, several different DNA template concentrations were tested and, on the basis of logistics as outlined by Jennings et al. (*16*) and the standardization carried out in our laboratory for the probability of transgene detection from animal tissues, 1 μg of DNA template was used in the PCR setup for transgene fragments. The limit of detection (LOD) for transgenic DNA was determined as described by Jennings et al. (*16*).

All DNA extracted from GI tract and organ tissues was subjected to PCR with primers specific for amplification of ovine or porcine DNA (as a positive animal-specific control), for Cp, a chloroplast-specific *rubisco* DNA fragment (plant control), and for amplification of *cp4 epsps* transgene fragments (for transgene detection). The DNA from digesta samples was also reacted against bacterial-specific primers as additional positive controls.

The primers used for PCR are presented in **Table 2** and are described below. To confirm the specificity of PCR products, the positive amplicons for *cp4 epsps* and *rubisco* were sequenced and showed homology (>98% homology within sequencing error rates) when compared with gene sequences for *rubisco* and *cp4 epsps*.

Plant-Specific Primers for Endogenous DNA. For GI tract tissues, small (186 bp) and large (540 bp) fragments of *rubisco* DNA (*rbc* SF and *rbc* LF, respectively) and chloroplast-specific DNA (Cp) were amplified. Endogenous canola DNA was detected by amplification of *rbc* LF (540-bp fragment) of *Brassica napus* ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*rubisco*; GenBank Accession no. X75334) using forward primer 5′-GCG TGA CGT CGT CAC GTA G- 3′ (LF) and reverse primer 5′-CGT TGC CTG CCA CAG GAT TAA GG-3′ (LR). Conditions for PCR were as follows: 95 °C for 5 min, 30 cycles of 94 °C for 20 s, 50 °C for 30 s, and 72 °C for 3 min, and a final extension for 10 min at 72 °C. A smaller *B. napus* intrinsic *rbc* SF fragment (186 bp) was detected using the primers and conditions described by Sharma et al. (*28*). A high-copy chloroplast-specific DNA (Cp) fragment was detected using forward primer CpF 5′-CC TGT TGA GCT TGA CTC TAG TCT GGC-3′ and reverse primer CpR 5′-AAG AGC CGA CAT CGA AGG ATC-3′. These primers, provided by the Lethbridge Research Centre potato research laboratory, were based on a highly homologous segment in the noncoding region between the *trnT* and *trnF* genes of the chloroplast DNA (*29*) and targeted an amplicon of 520 bp for *B. napus* DNA (as tested on canola leaves).

Plant-Specific Primers, Transgenic. Primer sets amplifying the 1.3-kb *cp4 epsps* whole gene, as well as five other smaller (≤300 bp) fragments (F1−F5) spanning the length of the recombinant *cp4 epsps* construct, were designed (*28*). It was hypothesized that using smaller amplicons would increase the chance of transgene fragment detection, given that whole gene has seldom been detected in the digesta/tissue. The construct-specific fragments F1, F2, and F5 were designed to prevent false positives by having at least one of the primers (forward or reverse) located in the promoter or terminator region of the gene.

For detection of the entire 1.3-kb *cp4 epsps* gene (F6), forward primer EF1 (5′-TCA CGG TGC AAG CAG CCG TCC AGC-3′) and reverse

primer ER1 (5′-TCA AGC AGC CTT AGT GTC GGA GAG TTC G-3′) were used, with PCR conditions as follows: 94 °C for 5 min, 74 °C for 5 min, 35 cycles of 94 °C for 1 min, 74 °C for 3 min, and 72 °C for 10 min.

Animal-Specific Primers. The identity of porcine tissue DNA was ascertained using forward primer PF 5′-GAA ACA ATT CCC GTG TTC TCT-3′ and reverse primer PR 5′-TCA CTT CCA CAC CTG TAA CAT CT-3′, which were specific for an amplicon of ~532 bp (*30*). As a positive control for ovine tissue, a 652-bp fragment was amplified using primers OF 5′-CAA CAG GAAGGA ATC ATT ACA GTA-3′ and OR 5′-CCA AAA CAG CCG CTT ATC CAA G-3′ as described previously (*31*).

Bacteria-Specific Primers. For digesta samples, universal primers were used to detect the bacterial DNA fragment (Bc) encoding 16S rDNA (*32*) with previously standardized primer sequences and thermocycling conditions (*23*).

Agarose Gel Electrophoresis. Each PCR product (extracted DNA and appropriate controls) was analyzed by electrophoretic separation of a 20-μL aliquot on a 1.5% (w/v) agarose gel containing ethidium bromide (*33*).

Southern Hybridization. Samples testing negative by PCR were processed for Southern blotting and hybridization to confirm the absence of the target DNA sequences. For this purpose, electrophoretically separated PCR products (20 μL) were blotted onto Zeta-Probe membranes (Bio-Rad, Mississauga, ON) as described by Koetsier et al. (*34*). After blotting, the membranes were air-dried and UV cross-linked using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). The membranes were either processed directly for hybridizations or stored between two sheets of 3MM Whatman cellulose chromatography paper (Fisher Scientific Limited, Nepean, ON) in plastic containers.

Probe preparation and Southern hybridization were carried out using the AlkPhos Direct labeling kit (Amersham Biosciences, Piscataway, NJ). The DNA for probe preparation was obtained by PCR amplification (100-μL reaction) of DNA from RR canola leaf DNA. Depending on the primers that were positive by PCR, either the entire 1.3-kb *cp4 epsps* gene (F6), fragment F1 (promoter/chloroplast transit peptide region), or fragment F5 (*cp4 epsps*/terminator region) was amplified. A 5-μL aliquot of the PCR product assessed by electrophoresis on a 1.5% agarose gel, and the remaining product was cleaned using a QIAquick Gel Extraction kit (Qiagen). Following spectrophotometric quantification, the gel-eluted DNA was diluted to a concentration of 10 ng/μL. The diluted DNA was heat-denatured in boiling water (5 min) and then snap-cooled on ice. An equal volume of reaction buffer (supplied in the kit) was added to the diluted DNA, the probe was prepared according to the manufacturer's instructions, and aliquots were stored in 50% (v/v) glycerol at −20 °C for a maximum of 2 months.

The blotted membranes were incubated in prewarmed (55 °C) hybridization solution for a minimum of 4 h prior to the addition of labeled probe. Typically, 25 μL of probe was added to the hybridization tube for a 15 × 10 cm membrane, but the amount of probe added was adjusted depending on the area of the blotted membrane. Hybridization was carried out overnight at 60 °C, and membranes were washed with primary and secondary washes recommended by the manufacturer. Upon completion of the secondary washes, the membranes were placed in Saran plastic wrap, 700 μL of detection reagent was added, and membranes were exposed to Fuji X-ray film (Fuji, Tokyo, Japan) for 2 h, typically, with exposure time adjusted depending upon the signal strength.

Data Analysis. The results of a PCR were accepted when the positive control was positive and the negative control was negative for the amplicon under investigation. PCR was repeated if results from the duplicate PCR analyses were ambiguous or not in agreement (+/− or −/+). Observations from each tissue sample were then recorded as (results of PCR conducted on first extraction) (results of PCR conducted on second extraction). At this point, the samples with observations recorded as + +, + −, or − + were classified as positive, and those with PCR observations of − − were classified as tentatively negative. Samples tentatively negative by PCR (or ambiguously positive, when applicable) were analyzed further by Southern blotting and hybridization for definitive classification. Final classifications of samples required that PCR results were consistent (+ + or − −) between the duplicate extractions. Samples yielding + + are recorded as positive values in **Tables 3** and **4**.

Table 3. Detection[a] of Endogenous and Transgenic Plant DNA Fragments in Digesta and Tissue Samples from Lambs Fed Diets Containing Roundup Ready Canola ($n = 11$)

fragment amplified	digesta samples[b]			tissue samples[c]					organ tissue	
	R	A	I	ES	RU	AB	SI	LI	liver	kidney
endogenous[d]										
rbc LF	4	2	5	0	0	0	1	2	0	0
rbc SF	4	9	8	0	1	2	3	2	1	0
Cp	11	11	11	1	5	8	11	10	3	2
transgenic[d]										
F1	1	2	6	0	1	2	3	3	0	0
F2	2	5	6	0	1	0	1	2	0	0
F3	4	4	7	0	3	1	1	3	0	0
F4	1	2	3	0	2	1	2	1	0	0
F5	2	2	7	0	0	1	1	1	0	0

[a] Number of positive samples of 11 collected and analyzed (1 from each of the 11 lambs fed). When PCR data were ambiguous or apparently negative, Southern blot analysis was conducted for confirmation of findings. [b] For digesta samples: R, ruminal content; A, abomasal content; I, large intestinal content. [c] For tissue samples: ES, esophagus; RU, rumen; AB, abomasum; SI, small intestine; LI, large intestine. [d] Primers, amplicon size, and description of *rbc* LF, *rbc* SF, Cp, and fragments F1–F5 from the *cp4 epsps* transgene are outlined in **Table 1**.

Table 4. Detection[a] of Endogenous and Transgenic Plant DNA Fragments in Digesta (Cecal Content) and Tissue Samples from Pigs Fed Diets Containing Roundup Ready Canola ($n = 36$)

fragment amplified	cecal content	GI tract tissues		organ tissue		
		duodenum	cecum	liver	spleen	kidney
endogenous[b]						
rbc LF	20	6	8	0	0	1
rbc SF	29	8	9	0	1	1
Cp	36	32	33	1	4	2
transgenic[b]						
F1	22	2	2	0	0	0
F2	14	0	2	0	0	0
F3	27	1	5	1	0	1
F4	12	4	10	0	0	0
F5	23	0	5	0	0	0

[a] Number of positive samples of 36 collected and analyzed (1 from each of the 36 pigs fed). When PCR data were ambiguous or apparently negative, Southern blot analysis was conducted for confirmation of findings. [b] Primers, amplicon size, and description of *rbc* LF, *rbc* SF, Cp, and fragments F1–F5 from the *cp4 epsps* transgene are outlined in **Table 1**.

RESULTS AND DISCUSSION

DNA Analyses. Digesta samples were found to contain significant PCR inhibitors, which necessitated modification of the extraction protocols to remove these inhibitors without compromising DNA yield (*26*). The protocols eventually defined, as described herein, enabled extraction of DNA of reasonable purity from the different tissues and digesta.

PCR Analyses. To overcome potentially ambiguous findings and misinterpretations of data arising from possible false negatives or positives, separate duplicate DNA extractions of each sample were conducted, and each extracted DNA was subjected to duplicate, independent PCR. Following the strategy outlined by Jennings et al. (*16*), consistency of results between duplicate extracts was prerequisite for reporting.

In instances when visualization of PCR products was difficult, either because of faint bands or small amounts of PCR product, Southern blotting was conducted for unequivocal confirmation. Because the probe binding was very specific, exposure of the

film for <2 h in most cases produced a signal that enabled visualization of PCR product (if present). The blots were overexposed, however, to increase sensitivity in confirming negative findings. Positive results from spiked samples and RR canola leaf genomic DNA template were conditions of acceptance of extraction and PCR data. If appropriate controls (animal- or bacteria-specific DNA) were not amplifiable in extracted DNA, the extraction was repeated using the third subsample.

Limits of detection of gene fragments by PCR were found to be 50 pg for DNA from tissue and 25 pg for DNA from digesta. When Southern hybridization procedures were used, the LOD was routinely <1 pg, which is less than that for the haploid genome of canola (1.2 pg). This level of sensitivity (i.e., less than that of the haploid genome) has been used previously as a threshold for detection of transgenic fragments from RR soybean (*16*).

Extracted DNA purified from the experimental diets was determined previously to have molecular size in the range of 23 kbp (*23*), which is consistent with the presence of intact DNA. The presence of *cp4 epsps* in Roundup Ready GM canola leaves, seeds, meals, and diets and the absence of transgene in the near-isogenic parental line (leaves, meals, and diets) were confirmed by PCR prior to the start of the trial (*23*, Figure 2). Low-copy *rubisco* (*rbc* LF and SF) as well as high-copy chloroplast-specific DNA fragment (Cp) was detected in both the RR and PAR diets.

It is widely recognized that detectability of plant gene fragments is a function of copy number (*35*). Depending on the tissue type, a plant cell may contain 500−50000 copies of the chloroplast genome, in sharp contrast to the single copy of transgene DNA in a nuclear genome (*36*), and would thus have a much higher probability of detection, even from GM crop-derived diets. Detection of high-copy chloroplast-specific gene fragments should therefore not be taken as a sign of transgene presence. For this reason, the *rubisco* gene was included in the present study as a positive control in addition to the high-copy chloroplast-specific DNA fragment (Cp).

Sheep Trial. *Detection of DNA in Digesta.* The high-copy chloroplast-encoded Cp fragment was detected in ruminal, abomasal, and large intestinal content from all 11 lambs (**Table 3**) and consistently produced a high-intensity band (**Figure 1a**). This observation varies slightly from the real-time PCR data reported by Einspanier et al. (*37*), who found that ubiquitious plant chloroplast DNA fragment persisted more frequently in rumen and abomasal ingesta, compared to content from the lower GI tract (colon). The *rbc* fragments (nuclearly encoded; **Figure 1b**,**c**) were detected less frequently than Cp, which is reflective of their relative copy numbers. The *rbc* LF amplicon was detected in 4, 2, and 5 of the 11 ruminal, abomasal, and intestinal samples, respectively, compared with 4, 9, and 8 samples positive for the smaller *rbc* SF amplicon (**Table 3**). Similarly, small fragments of *rubisco* DNA (high copy number) were detected in duodenal chyme from cows fed recombinant (Bt) maize silage (*10*), and Chowdhury et al. (*38*) reported the detection of *rubisco*, chloroplast tRNA, and *cry1Ab* DNA fragments in ruminal fluid and rectal contents of maize-fed calves. In the present study, the smaller *rubisco* fragment (*rbc* SF) was detected more frequently in abomasal content than in ruminal digesta (9 of 11 versus 4 of 11).

As expected, the 466-bp bacterial DNA fragment (**Figure 1d**) was amplifiable in all digesta samples from each of the sheep, attributable to the high density of bacterial cells in the GI tract.

Transgene Fragments in Digesta. The frequency of detection of the *cp4 epsps* transgene fragments in digesta samples from the 11 lambs ranged from 9 to 63% (**Table 3**), with F3 detected most frequently overall. This indicates that plant DNA that had persisted despite the degradative effects of the mixing and pelleting processes (*23*) was still not completely degraded in the GI tract. In an earlier study, we determined that free DNA from the diets was detectable for only 10 min of incubation in ruminal fluid filtrate (*3*). Others have also reported rapid degradation of free DNA in contact with digestive fluids (*39*, *40*). The DNA detected here is likely that associated with plant cells that afforded some measure of protection against nuclease activity.

The persistence of DNA in vivo exceeded that observed earlier in vitro. In the present study, each of the five *cp4 epsps* fragments was detected at least once in each of the three digesta types (**Table 3**). This finding supports our earlier hypothesis (*23*) that although plant DNA released during plant cell degradation is rapidly degraded by nuclease activity in the digestive tract, some feed-ingested DNA does escape this hydrolysis in whole digesta, most likely through association with undigested plant material (intact cells). The hypothesis and present observations are also consistent with findings from an earlier study in which bovine ruminal and duodenal digesta were separated into solid and liquid fractions (*4*). In that study, high-copy bovine mitochondrial cytochrome *b* and *rubisco* genes were detectable in the majority of both phases of digesta, but fragments of single-copy genes encoding lectin and corn high-mobility protein, as well as *cp4 epsps* and *cry1a(b)*, could not be amplified in the liquid phases of either ruminal or duodenal digesta. This was attributed to extremely rapid DNA degradation in the liquid phase (*3*, *39*, *40*).

It was expected that DNA would be degraded by DNases secreted by salivary glands and by Paneth cells in the small intestine (*41*), yet transgene fragments were detected in digesta from the large intestine as well as from the upper digestive tract. Intense degradation of DNA in the rumen and abomasum may have led to low rates of detection, whereas in the intestine, food particles become more concentrated and degradation is known to be less severe (*8*). The concentration of undigested whole plant cells would also be anticipated to increase in the lower digestive tract. Tony et al. (*12*) also observed incomplete degradation of Bt 176 maize DNA in the broiler GI tract, and, similarly, Chowdhury et al. (*38*) have recently reported *cry1Ab* gene detection at different frequencies from abomasal, jejunal, and cecal contents of calves fed Bt11 maize.

Detection of DNA in Ovine Tissues. The ovine-specific primers (OF/OR) selected for positive controls amplified a 652-bp fragment (**Figure 2a**,**b**) that confirmed the DNA from ovine tissues was in every case of suitable quality for PCR. From preliminary studies to refine PCR conditions (**Figure 2a**), protocols including 5 μg of BSA in the PCR mixture and template DNA in amounts of 200 ng for tissue controls (OF/OR), 100 ng for plant controls (leaf tissue), and 1 μg for detection of transgene fragments were adopted as standard.

The high-copy chloroplast-encoded Cp fragment was detected in ovine GI tissues (**Figure 2c**) with widely ranging frequencies. This fragment was amplifiable in only 1 of 11 samples from the esophagus compared with 10 and 11 of the small and large intestinal tissues, respectively (**Table 3**). Low-copy *rbc* LF (**Figure 2d**) and *rbc* SF were detected at much lower frequency than Cp, but, similar to Cp, were more common in intestinal tissues than in the upper GI tract. Faint banding patterns that were observed by gel electrophoresis of PCR products (e.g.,

lanes 2 and 3, **Figure 2d**) were confirmed subsequently by Southern hybridization.

Endogenous plant DNA fragments could be amplified in DNA from ovine liver and kidney tissues (**Table 3**), although less frequently than in tissues from the GI tract. In contrast, Einspanier et al. (*10*) detected no high-copy chloroplast-specific DNA fragments in muscle, liver, or spleen of cattle. In the ovine liver and kidney tissues in the present study, the Cp fragment was slightly more common (5 of 22 samples) than were *rbc* SF or *rbc* LF (present in 1 and 0 samples, respectively). A *rubisco* gene fragment (231 bp) and tRNA fragment (196 bp) were also detected occasionally from liver, spleen, and kidney tissues of calves fed Bt11 maize (*38*).

Transgene Fragments in Ovine Tissue. Fragments of the *cp4 epsps* transgene were detected at low frequencies among ovine GI tract tissues, except the esophagus, but not in any of the liver or kidney samples (**Figure 2e**−**g**; **Table 3**). Other researchers have also failed to detect transgene (*cry1Ab*) fragments in visceral tissues from calves fed Bt11 maize (*10*, *38*, *42*), although the Cry1Ab protein was found in digesta (*42*). It is possible that the plant DNA (endogenous or transgenic) detected in GI tract tissue in the present study may have arisen from small amounts of digesta adhering to the tissue samples (although care was taken to wash them thoroughly with saline solution prior to storage), but the detection of DNA in tissue as a result of uptake by epithelial cells in the GI tract cannot be ruled out.

Patterns of detection of DNA fragments in samples collected in this study support the hypotheses that likelihood of detection is related to copy number of the gene (e.g., high-copy Cp fragments versus low-copy *rbc* and *cp4 epsps* fragments) and that likelihood of uptake of feed-ingested DNA into tissues is related to exposure of epithelial tissues to undegraded, free DNA (e.g., virtually no plant DNA detected in esophageal tissue; more frequent detection in distal GI tract tissues, where intestinal degradation of plant cells would effect release of DNA previously protected from nucleases in digesta).

Swine Trial. *Detection of DNA in Cecal Contents.* Chloroplast-specific DNA (Cp) fragment was detected in cecal content from all 36 of the pigs tested (**Table 4**), similar to the detection of high-copy *rubisco* in cecal samples from 10 of 10 pigs fed Bt-11 corn (*43*). As was observed with lamb digesta in the present study, in porcine cecal content, the Cp fragment was detected more frequently than were *rbc* LF and SF (**Table 4**). The smaller *rubisco* fragment (*rbc* SF) was again slightly more common than the large fragment (*rbc* LF). Nemeth et al. (*35*) also detected a 173-bp maize *rbcL* fragment with slightly higher frequency than the larger (500-bp) *rbcL* gene (53 versus 43%) in porcine muscle samples. As observed with digesta from sheep, the 466-bp bacterial DNA fragment was amplifiable in each of the 36 cecal content samples (data not shown).

Transgene Fragments in Cecal Content. All of the *cp4 epsps* transgene fragments were detectable in cecal content from at least some of the pigs (**Table 4**), with frequencies ranging from 75% (F3; **Figure 3a**) to 33% (F4; **Figure 3b**). This is consistent with other detection frequencies (25−75%) for similarly sized *cry1Ab* and *cry9C* transgene fragments (103−532 bp) in the content of GI tracts of cattle and pigs fed Bt-corn (*10*, *19*, *38*, *43*, *44*). Some studies, however, have reported nondetection of recombinant DNA fragments in digesta or GI tract contents, particularly in monogastric animals [reviewed by Flachowsky et al. (*45*)]. For example, Klotz et al. (*17*) reported a time-dependent detection of chloroplast-specific DNA in the GI tract contents of pigs but found no *cry1A* transgene. This was attributed to its low copy number. Using real-time PCR, Einspanier et al. (*37*) recently detected chloroplast DNA (high copy) in the GI tract of cattle fed Bt-maize, but low-copy fragments of transgene *cry1Ab* were not observed.

The disparities detection of recombinant DNA among studies such as these may be linked to the amount and type of feed provided, which directly determines the starting quantity of transgene consumed. In a recent study, the transgene in Bt-corn was shown to decrease to only 3% of its initial concentration during the ensiling process (*37*). Digestibilities of diets may also impact the persistence and therefore detectability of feed-ingested DNA, including transgenes. Intact plant cells afford protection to genetic material as feed passes through a number of hostile environments. For example, fragments of *cry1Ab* were detectable for only 30 min in silage effluent and for only 60 min in ovine saliva (*39*). More severe feed-processing techniques will also increase plant cell lysis and effect release of larger quantities of DNA. Furthermore, there is ample evidence of degradation of free DNA in ruminant and monogastric GI tracts (*41*, *45*, *46*) upon its release from partially or completely digested feeds. Through acid hydrolytic depurination of adenine and guanine nucleotides (*47*) and enzymatic digestion, DNA is cleaved into small fragments and free nucleotides (*41*, *46*), with further degradation by deaminase and acid/alkaline phosphatase activities along the digestive tract. Nonetheless, transgenes do persist, as evidenced by detection of *cp4 epsps* fragments in cecal content of pigs in the present study.

Detection of DNA in Porcine Tissues. Porcine-specific primers (PF/PR) were used as a positive control for DNA extracted from tissues and consistently amplified a 532-bp fragment (**Figure 3c**). The high-copy chloroplast Cp fragment was detected in the majority of duodenal and cecal tissues (**Table 4**), but as observed in sheep, detection of *rbc* LF and SF was less common, with frequencies of ≤25%. In agreement with the findings of Reuter and Aulrich (*19*), endogenous plant DNA was detected in visceral organ tissues (liver, spleen, kidney) of pigs in the present study, although considerably less frequently than was observed by those researchers. In our study, the Cp fragment was detected in only 7 of 108 samples assessed (36 animals × 3 sample types; **Table 4**) and *rbc* in only 2 (SF) or 1 (LF) of the 108. This disparity may be related to differences in the size of the fragments amplified. Reuter and Aulrich (*19*) amplified a 140-bp chloroplast gene fragment, compared with the 540-bp Cp fragment used in the present study.

Transgene Fragments in GI Tissues. Fragments of the *cp4 epsps* transgene were detected in porcine GI tissues with frequencies ranging from 0 to 28% (**Table 4**). Detection was more common in cecal than in duodenal tissues. Interestingly, fragment F4, which was observed least frequently in cecal digesta, was most commonly detected in cecal tissue. Again, the possibility of positive findings in GI tissue having arisen from contamination with digesta cannot be discounted, but uptake of feed-ingested transgene (as for endogenous plant DNA) through the gut epithelium remains a viable explanation of these observations. Absorption/uptake of genetic material from the digesta is dependent upon the amount, degradation status, and residence time of the DNA in the GI tract and juxtaposition of the fragments and the gut epithelium.

Transgene Fragments in Organ Tissues. Transgene fragments F1, F2, F4, and F5 were not detected in any of the liver, kidney, or spleen tissues tested (**Table 4**; representative data in **Figure 3d**,**e**). Similarly, detection of plant chloroplast gene, but not of transgene, in the liver, spleen, and kidney has been reported for *Bt* in chickens (*12*) and pigs (*19*) fed GM maize. A novel

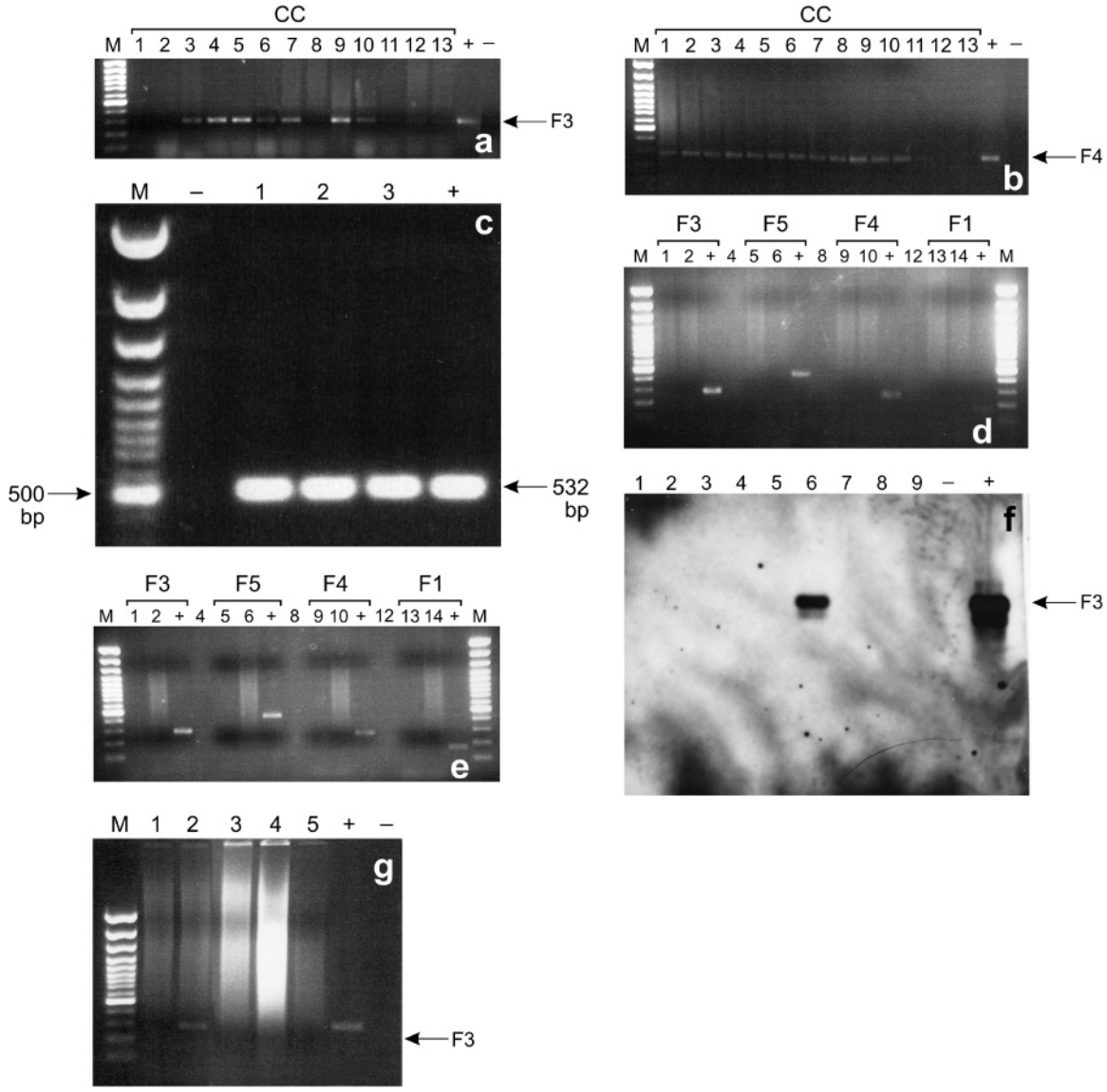

Figure 3. Representative electrophoretic gels and Southern hybridizations of PCR analyses of digesta and tissues from 36 pigs fed finisher diets containing 15% Roundup Ready canola meal: (**a**, **b**) detection of *cp4 epsps* fragment F3 (**a**) or fragment F4 (**b**) in cecal contents from 13 of the 36 pigs studied; (**c**) amplification of a 532-bp fragment of the gene encoding porcine growth factor in duodenal tissue from three pigs, demonstrating PCR readiness of the extracted DNA [the positive control (lane +) contains DNA extracted from kidney tissue of a pig fed non-GM canola]; (**d**, **e**) representative negative findings for detection of *cp4 epsps* transgene fragments F1, F3, F4, and F5 in liver (**d**) and kidney (**e**) tissues from two different pigs (lanes 4, 8, and 12 are empty); (**f**) the single positive (lane 6) and 8 (lanes 1–5 and 7–9) of 35 negative findings for detection of the *cp4 epsps* transgene fragment F3 in porcine liver tissue by Southern blot and hybridization; (**g**) the single positive (lane 2) and 4 (lanes 1 and 3–5) of 35 negative findings for *cp4 epsps* transgene fragment F3 in porcine kidney tissue by gel electrophoresis of PCR product. In each panel, lane M contains a 100-bp DNA ladder. The negative control lanes (–) contain no template DNA.

finding of the present study, however, is the detection of *cp4 epsps* transgene fragment F3 in porcine liver and kidney (**Figure 3f**,**g**). This fragment was found in one sample of each tissue type, collected from two different pigs. The positive PCR results from the kidney tissue were confirmed by Southern hybridization. The positive liver sample was detected only during routine Southern follow-up analysis of samples negative by PCR, which indicates that the fragment was present in very low quantities. In contrast, Jennings et al. (*16*) detected no *cp4 epsps* in muscle tissue from pigs fed diets containing 24% RR soybean, but in that study, PCR was conducted only on muscle tissue, as opposed to visceral organs.

Following these initial novel findings, repeat analyses with numerous PCR and hybridization variations and controls were conducted on extractions from the same two subsamples of the positive tissues, and initial observations were confirmed. As additional follow-up, DNA was extracted from the third subsamples that had been stored, but PCR and Southern hybridization consistently revealed both of those subsamples to be negative for fragment F3. This constancy of findings within subsample suggests that the positive results did not arise from contamination during extraction or PCR setup. Given the sensitivity of the assay, the possibility of incidental acquisition of transgenic DNA during processing cannot be discounted, but on the other hand, detection of low-copy DNA fragments in visceral tissue was also demonstrated with *rbc* SF and LF fragments, also at low frequency. Thus, this study may represent a random detection of transgene in 2 of the 108 visceral tissues assessed.

Detection of transgenic DNA fragments in visceral tissues in the present study may have been facilitated by the relatively large number of animals surveyed ($n = 36$). The likelihood of

transgene detection may also have been increased by our having used 2-g subsamples for extraction of DNA. These were large in comparison to other studies, in which 1-g (*35*), 100−300-mg (*10*), 25-mg (*19*), or 20-mg (*38*) samples were extracted. The fact that only one (F3) of the five possible *cp4 epsps* fragments was detected argues against contamination having arisen from DNA carry-over. Interestingly, F3 was the most commonly detected of the five fragments in digesta, which may have been related to a greater propensity for uptake and possible transfer into tissues. Mechanisms for such a transfer into blood leukocytes and tissues through M cells on Peyer's patches have been suggested previously by Chowdhury et al. (*38*), and intensive study has also been conducted by Schubbert et al. (*5*, *6*).

The nonuniformity of results across the three subsamples could be attributable to unequal distribution of plant DNA within organ tissues. Genomic libraries were prepared from the positive liver and kidney tissues for assessment of flanking sequences using the Universal Genome Walker kit (Clontech, Mountain View, CA). This technique allows amplification of an unknown region of DNA adjacent to a known region. In the present case, however, results were not conclusive, and integration of F3 was not confirmed. Thus, even though liver and kidney tissue samples tested positive for transgenic fragment F3 in this study, no evidence of integration of the transgenic fragments into porcine (mammalian) DNA was obtained. It cannot be discounted, therefore, that this genetic material was detected upon having entered these organs via the circulatory or lymphatic systems. From the procedures employed in this study, we were not able to ascertain if F3 was intracellular or extracellular (interstitial) within the positive liver or kidney tissues. Detection of this fragment in tissue from swine but not lambs may be a reflection of greater activity of microbial nucleases in the ruminant digestive tract as compared with monogastric.

The present study suggests that the likelihood of uptake of transgenic plant DNA into organ tissues of ruminant or monogastric animals, although possible, is low and adds to a growing body of evidence supporting such a conclusion. In human ileostomists consuming up to 454 g of GM soya, the proportion of transgene recoverable via the stoma did not exceed 3.7% during the 6 h immediately after intake (*21*). In that study, no transgene survived passage through the intact human GI tract. The World Health Organization (WHO) has declared no inherent risk in consuming DNA from GM crops (*1*). Evidence indicating substantive equivalence of GM crops [including RR canola, Stanford et al. (*24*)] with regard to nutritional value and animal performance continues to mount (*45*, *48*). It would appear, therefore, that the agronomic and nutritional benefits of GM crops for feed or food would outweigh any perceived risk of uptake or integration of transgenes.

This study investigated the passage of transgenic DNA through the ruminant and nonruminant (monogastric) digestive tracts using lambs and pigs fed GM canola meal under conditions typical of commercial livestock production. Native and transgenic DNA exhibited similar stability and persistence in the gastrointestinal tract. Fragments of both DNA types were detected in intestinal digesta, available for uptake into animal tissue. The techniques used enabled demonstration of uptake of low-copy endogenous and transgenic DNA fragments in gastrointestinal tract tissues of both lambs and pigs, and for the first time, uptake of transgene fragments into visceral tissue (kidney, liver) in pigs was also observed.

ACKNOWLEDGMENT

We thank R. Coleman and S. Cook for technical support and K. Jakober for assistance with the manuscript. The primers CpF/CpR were a gift from L. Lintott (potato research laboratory at Lethbridge Research Centre). We gratefully acknowledge provision of parental and Roundup Ready seeds, meals, and recombinant gene sequences by Monsanto Co.

LITERATURE CITED

(1) FAO/WHO. *Safety Aspects of Genetically Modified Foods of Plant Origin*; report of a Joint FAO/WHO Expert Consultation on food derived from biotechnology; FAO/WHO: Geneva, Switzerland, 2005; http://www.who.int/foodsafety/biotech/who_study/en/index.html.

(2) European Commission. EU official documents. Questions and answers on the regulation of GMOs in the EU, Brussels, Belgiuim, July 24, 2001; available online at http://europa.eu.int/comm/dgs/health_consumer/library/press/press171_en.pdf.

(3) Alexander, T. W.; Sharma, R.; Deng, M. Y.; Whetsell, A. J.; Jennings, J. C.; Wang, Y.; Okine, E.; Damgaard, D.; McAllister, T. A. Use of quantitative real-time and conventional PCR to assess the stability of the *cp4 epsps* transgene from Roundup Ready canola in the intestinal, ruminal, and fecal contents of sheep. *J. Biotechnol.* **2004**, *112*, 255−266.

(4) Phipps, R. H.; Deaville, E. R.; Maddison, B. C. Detection of transgenic and endogenous plant DNA in rumen fluid, duodenal digesta, milk, blood, and feces of lactating dairy cows. *J. Dairy Sci.* **2003**, *86*, 4070−4078.

(5) Schubbert, R.; Lettmann, C.; Doerfler, W. Ingested foreign (phage M13) DNA survives transiently in the gastrointestinal tract and enters the bloodstream of mice. *Mol. Gen. Genet.* **1994**, *242*, 495−504.

(6) Schubbert, R.; Renz, D.; Schmitz, B.; Doerfler, W. Foreign (M13) DNA ingested by mice reaches peripheral leukocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 961−966.

(7) Schubbert, R.; Hohlweg, U.; Renz, D.; Doerfler, W. On the fate of orally ingested foreign DNA in mice: chromosomal association and placental transmission to the fetus. *Mol. Gen. Genet.* **1998**, *259*, 569−576.

(8) Wilcks, A.; van Hoek, A. H.; Joosten, R. G.; Jacobsen, B. B.; Aarts, H. J. Persistence of DNA studied in different ex vivo and in vivo rat models simulating the human gut situation. *Food Chem. Toxicol.* **2004**, *42*, 493−502.

(9) Ash, J. A.; Scheideler, E.; Novak, C. L. The fate of genetically modified protein from Roundup Ready soybean in the laying hen. *Poult. Sci.* **2000**, *79* (Suppl. 1), 26 (Abstr. 111).

(10) Einspanier, R.; Klotz, A.; Kraft, J.; Aulrich, K.; Poser, R.; Schwägele, F.; Jahreis, G.; Flachowsky, G. The fate of forage plant DNA in farm animals: a collaborative case-study investigating cattle and chicken fed recombinant plant material. *Eur. Food Res. Technol.* **2001**, *212*, 129−134.

(11) Jennings, J. C.; Albee, L. D.; Kolwyck, D. C.; Surber, J. B.; Taylor, M. L.; Hartnell, G. F.; Lirette, R. P.; Glenn, K. C. Attempts to detect transgenic and endogenous plant DNA and transgenic protein in muscle from broilers fed YieldGard Corn Borer corn. *Poult. Sci.* **2003**, *82*, 371−380.

(12) Tony, M. A.; Butschke, A.; Broll, H.; Grohmann, L.; Zagon, J.; Halle, I.; Danicke, S.; Schauzu, M.; Hafez, H. M.; Flachowsky, G. Safety assessment of Bt 176 maize in broiler nutrition: degradation of maize-DNA and its metabolic fate. *Arch. Anim. Nutr.* **2003**, *57*, 235−252.

(13) Faust, M. A. Livestock products: composition and detection of transgenic DNA/proteins. *Proc. Symp. Agric. Biotechnol. Market*; ADAS-ASAS: Baltimore, MD, 2000; p 29.

(14) Phipps, R. H.; Beever, D. E.; Tingey, A. P. Detection of transgenic DNA in bovine milk: results for cows receiving TMR containing Bt insect protected maize grain (MON 810). *J. Ani. Sci.* **2001**, *79* (Suppl. 1), 114 (Abstr. 476).

(15) Weber, T. E.; Richert, B. T. Grower-finisher growth performance and carcass characteristics including attempts to detect transgenic plant DNA and protein in muscle from pigs fed genetically modified Bt corn. *J. Anim. Sci.* **2001**, *79* (Suppl. 2), 67.

(16) Jennings, J. C.; Kolwyck, D. C.; Kays, S. B.; Whetsell, A. J.; Surber, J. B.; Cromwell, G. L.; Lirette, R. P.; Glenn, K. C. Determining whether transgenic and endogenous plant DNA and transgenic protein are detectable in muscle from swine fed Roundup Ready soybean meal. *J. Anim. Sci.* **2003**, *81*, 1447−1455.

(17) Klotz, A.; Mayer, J.; Einspanier, R. Degradation and possible carry over of feed DNA monitored in pigs and poultry. *Eur. Food Res. Technol.* **2002**, *214*, 271−275.

(18) Phipps, R. H.; Beever, D. E.; Humphries, D. J. Detection of transgenic DNA in milk from cows receiving herbicide tolerant (CP4EPSPS) soyabean meal. *Livest. Prod. Sci.* **2002**, *74*, 269−273.

(19) Reuter, T.; Aulrich, K. Investigations on genetically modified maize (Bt-maize) in pig nutrition: fate of feed-ingested foreign DNA in pig bodies. *Eur. Food Res. Technol.* **2003**, *216*, 185−192.

(20) Martìn-Oàue, S. M.; O'Donnell, A. G.; Ariňo, J.; Netherwood, T.; Gilbert, H. J.; Mathers, J. C. Degradation of transgenic DNA from genetically modified soya and maize in human intestinal simulations. *Br. J. Nutr.* **2002**, *87*, 533−542.

(21) Netherwood, T.; Martìn-Oŕue, S. M.; O'Donnell, A. G.; Gockling, S.; Graham, J.; Mather, J. C.; Gilbert, H. J. Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. *Nat. Biotechnol.* **2004**, *22*, 204−209.

(22) Canadian Council on Animal Care. In *Guide to the Care and Use of Experimental Animals*; Olfert, E. D., Cross, B. M., McWilliams, A. A., Eds.; CCAC: Ottawa, ON, Canada, 1993; Vol. 1.

(23) Alexander, T. W.; Sharma, R.; Okine, E. K.; Dixon, W. T.; Forster, R. J.; Stanford, K.; McAllister, T. A. Impact of feed processing and mixed ruminal culture on the fate of recombinant EPSP synthase and endogenous canola plant DNA. *FEMS Microbiol. Lett.* **2002**, *214*, 263−269.

(24) Stanford, K.; Aalhus, J. L.; Dugan, M. E. R.; Wallins, G. L.; Sharma, R.; McAllister, T. A. Effect of feeding transgenic canola on apparent digestibility, growth performance and carcass characteristics of lambs. *Can. J. Anim. Sci.* **2003**, *83*, 299−305.

(25) National Research Council. *Nutrient Requirements of Swine*, 10th ed.; National Academy Press: Washington, DC, 1998.

(26) Sharma, R.; John, S. J.; Damgaard, D. M.; McAllister, T. A. Extraction of PCR-quality plant and microbial DNA from total rumen contents. *BioTechniques* **2003**, *34*, 92−97.

(27) Kreader, C. A. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Appl. Environ. Microbiol.* **1996**, *62*, 1102−1106.

(28) Sharma, R.; Alexander, T. W.; John, S. J.; Forster, R. J.; McAllister, T. A. Relative stability of transgene DNA fragments from genetically modified canola in mixed ruminal cultures. *Br. J. Nutr.* **2004**, *91*, 673−681.

(29) Taberlet, P.; Gielly, L.; Pautou, G.; Bouvet, J. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol. Biol.* **1991**, *17*, 1105−1109.

(30) Mendez, E. A.; Messer, L. A.; Larsen, N. J.; Robic, A.; Rothschild, M. F. Epidermal growth factor maps to pig chromosome 8. *J. Anim. Sci.* **1999**, *77*, 494−495.

(31) Lanneluc, I.; Mulsant, P.; Harrison, B.; Drinkwater, R. D. Denaturing gradient gel electrophoresis detection of polymorphism in a PCR fragment of sheep epidermal growth factor gene. *Anim. Genet.* **1996**, *27*, 99−101.

(32) Nadkarni, M. A.; Martin, F. E.; Jacques, N. A.; Hunter, N. Determination of bacterial load by real-time PCR using a broad range (universal) probe and primer set. *Microbiology* **2002**, *148*, 257−266.

(33) Sambrook, J.; Fritsch, E. F.; Maniatis, T. Gel electrophoresis of DNA. In *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Ford, N., Nolan, C., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989; Vol. 1, pp 6.9−6.13.

(34) Koetsier, P. A.; Schorr, J.; Doerfler, W. A rapid optimized protocol for downward alkaline southern blotting of DNA. *BioTechniques* **1993**, *15*, 260−262.

(35) Nemeth, A.; Wurz, A.; Artim, L.; Charlton, S.; Dana, G.; Glenn, K.; Hunst, P.; Jennings, J.; Shilito, R.; Song, P. Sensitive PCR analysis of animal tissue samples for fragments of endogenous and transgenic plant DNA. *J. Agric. Food Chem.* **2004**, *52*, 6129−6135.

(36) Benedich, A. Why do chloroplasts and mitochondria contain so many copies of their genome? *BioEssays* **1987**, *6*, 279−282.

(37) Einspanier, R.; Lutz, B.; Rief, S.; Berezina, O.; Zverlov, V.; Schwarz, W.; Mayer, J. Tracing residual recombinant feed molecules during digestion and rumen bacterial diversity in cattle fed transgenic maize. *Eur. Food Res. Technol.* **2004**, *218*, 269−273.

(38) Chowdhury, E. H.; Mikami, O.; Murata, H.; Sultana, P.; Shimada, N.; Yoshioka, M.; Guruge, K. S.; Yamamoto, S.; Miyazaki, S.; Yamanaka, N.; Nakajima, Y. Fate of maize intrinsic and recombinant genes in calves fed genetically modified maize Bt11. *J. Food Prot.* **2004**, *67*, 365−370.

(39) Duggan, P. S.; Chambers, P. A.; Heritage, J.; Forbes, J. M. Survival of free DNA encoding antibiotic resistance from transgenic maize and the transformation activity of DNA in ovine saliva, ovine rumen fluid and silage effluent. *FEMS Microbiol. Lett.* **2000**, *191*, 71−77.

(40) Duggan, P. S.; Chambers, P. A.; Heritage, J.; Forbes, J. M. Fate of genetically modified maize DNA in the oral cavity and rumen of sheep. *Br. J. Nutr.* **2003**, *89*, 159−166.

(41) McAllan, A. B. The degradation of nucleic acids in and the removal of breakdown products from the small intestines of steers. *Br. J. Nutr.* **1980**, *4*, 99−112.

(42) Chowdhury, E. H.; Shimada, N.; Murata, H.; Mikami, O.; Sultana, P.; Miyazaki, S.; Yoshioka, M.; Yamanaka, N.; Hirai, N.; Nakajima, Y. Detection of Cry1Ab protein in gastrointestinal contents but not visceral organs of genetically modified Bt11-fed calves. *Vet. Hum. Toxicol.* **2003**, *45*, 72−75.

(43) Chowdhury, E. H.; Kuribara, H.; Hino, A.; Sultana, P.; Mikami, O.; Shimada, N.; Guruge, K. S.; Saito, M.; Nakajima, Y. Detection of corn intrinsic and recombinant DNA fragments and Cry1Ab protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11. *J. Anim. Sci.* **2003**, *81*, 2546−2551.

(44) Chowdhury, E. H.; Mikami, O.; Nakajima, Y.; Kuribara, H.; Hino, A.; Suga, K.; Hanazumi, M.; Yonemoch, C. Detection of genetically modified maize DNA fragments in the intestinal contents of pigs fed StarLink CBH351. *Vet. Hum. Toxicol.* **2003**, *45*, 95−96.

(45) Flachowsky, G.; Chesson, A.; Aulrich, K. Animal nutrition with feeds from genetically modified plants. *Arch. Anim. Nutr.* **2005**, *59* (1), 1−40.

(46) McAllan, A. B. The fate of nucleic acids in ruminants. *Proc. Nutr. Soc.* **1982**, *41*, 309−317.

(47) Klinedinst, D. K.; Drinkwater, N. R. Mutagenesis by apurinic sites in normal and ataxia telangiectasia human lymphoblastoid cells. *Mol. Carcinog.* **1992**, *6*, 32−42.

(48) Flachowsky, G.; Aulrich, K. Nutritional assessment of feeds from genetically modified organism. *J. Anim. Feed Sci.* **2001**, *10*, 181−194.

Received for review October 4, 2005. Revised manuscript received January 3, 2006. Accepted January 11, 2006. This study was conducted with financial support from the Canadian Food Inspection Agency (CFIA), the Alberta Agricultural Research Institute, and the Alberta Livestock Industry Development Fund (Joint Project 2002L053). This is Lethbridge Research Centre Contribution 38705057.

JF052459O